Example 1

Amplification detected in real time

In order to monitor the performance of a *Scorpions* primer in an homogeneous amplification reaction, a PCR was performed using primers which flank a polymorphism in the BRCA2 gene. The target sequence selected had previously been used for allelic discrimination of the two variants but was too short for real time detection (the probe failed to hybridise at 60°C- the lowest temperature in the thermocycling run). The (upper strand) probe entity was synthesised as part of a lower strand primer with a blocking HEG between the two functionalities. Target DNA could be selected to produce amplicon which would match or mismatch the probe.

Reaction conditions: After addition of template DNA, tubes were sealed and reactions were cycled under the following conditions: 20 min at 94°C to activate the Amplitaq Gold; and 40 cycles of {94°C for 45s, 60°C for 45s}. Reactions were performed in an ABI PRISM 7700 fluorescence PCR machine.

Results: See Figure 13. It is very clear that as amplicon accumulates, fluorescent signal is generated. There are several fluorescence readings at each timepoint and the sharp, stepwise nature of the signal increase reflects the rapid production of probe-target duplex in the early part of the thermocycle hold. This is due to the unimolecular mode of action of a Scorpion primer, which permits instantaneous recognition of an appropriate amplicon.

Example 2

Allelic discrimination

Materials and methods as above.

Results: See Figure 14. In this experiment, the probe matched or mismatched the amplicons at the polymorphic base. Both amplifications were equally efficient (as viewed by agarose gels [results not shown]), but the matched product was detected much more readily than the mismatched. This illustrates the strong specificity of the system even down to a single base change in the amplicon.

Example 3

Primer Titration

Materials and method, see above. Titration of the primer B2098 with its untailed equivalent (R186-98) was from 100% *Scorpions* to 10% *Scorpions*; total primer was constant at 500 nM.

Results: See Figure 15. At all ratios of *Scorpions*:untailed primer, reactions were clearly detectable on the ABI7700. Indeed, the Ct (the point at which signal crosses a threshold above "background") was identical regardless of the ratio of *Scorpions* to untailed primer indicating the same levels of priming effieciency throughout the series. The only variable was absolute fluorescence signal (as would be expected). The efficiency of this system is in marked contrast to available methods where higher concentrations of probe are required to drive kinetically the bimolecular probing event.

Example 4

Endpoint readings

Materials and methods: Reactions were set up as above but were carried out at two different magnesium concentrations (1.2 and 3.5 mM). DNAs of all three genotypes and a no template control (NTC) were used and the fluorescence was measured before and after amplification. Fluorescence numbers are the means of at least 6 separate readings from duplicate samples.

Results

Sample	CC		AC		AA		NTC	
Mg	1.2	3.5	1.2	3.5	1.2	3.5	1.2	3.5
Before	6396	3706	5700	2958	6157	3299	6257	3685
After	12144	10316	8614	6140	6818	4641	6616	4453
Change	5748	6610	2914	3182	661	1342	359	768

Fluorescence readings increased through the PCR in a target dependent manner. In fact the signals generated for heterozygotes are approximately half those for the CC

homozygotes and this may be useful for genotyping in a simple way or for analysis of heteroplasmy where the allele ratios vary more widely than 100:0, 50:50 or 0:100. In addition, the signals generated for mismatch targets were similar to background levels showing that although amplification had occurred, the probe was not efficiently hybridising unless there was a perfect match. Increasing the magnesium concentration decreased this discrimination but also ensured that the backgrounds were lower, presumably by promoting hybridisation in general.

In addition to observing these signal changes by fluorimeter, increased fluorescence could be detected by visual inspection of the tubes backlit by UV transilluminator. This is a remarkable observation since the FAM dye has an excitation optimum at ~490nm whereas the UV box illuminates at ~330-360nm. This means that the fluorescent yield was far from optimal and may be substantially improved by the use of more appropriate wavelengths.

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Example 5

Analysis of heteroplasmy by Scorpions

Reactions were set up as in Example 4, but template DNA was a standard quantity with varying admixtures of C homozygote to A homozygote: 100%:0%, 90:10, 50:50, 10:90, 0:100 and NTC. After 40 cycles of PCR, the FAM fluorescence readings were taken and the NTC subtracted from each. The data are shown in Figure 16.

Example 6

Comparative Performance of Scorpions

In order to examine the relative performance of Scorpions versus a bimolecular equivalent, the same amplicon and probe sequences were used in each format. The bimolecular format constituted 500 nM each of primers R186-98 and R187-98, plus 500 nM Molecular Beacon Z3702, while the unimolecular version contained B2098 and R187-98 each at 500 nM. Other reaction constituents were identical to previous experiments (with 1.2 mM Mg) and cycling was for 40 cycles as above. The results of these amplifications in real time with targets which are homozygous C, homozygous A, or heterozygous A/C are shown in Figure 17. Clearly, there was no substantial amplification above background for the reactions